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Cyclin-dependent kinase inhibitors

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Cell-cycle dysregulation is one of the cardinal characteristics of neoplastic cells. For this reason, small molecule inhibitors targeting cyclin-dependent kinases (CDKs), of which flavopiridol is a prototype, have been the focus of extensive interest in cancer therapy. In addition to inhibiting cell-cycle progression, these agents exhibit a variety of other activities, including the induction of cell death. Recently, several novel mechanisms of action have been ascribed to the CDK inhibitor flavopiridol, including interference with transcription, most likely through disruption of P-TEFb (i.e. the CDK9/cyclin T complex), and induction of apoptosis, possibly a consequence of downregulation of various anti-apoptotic proteins. It has also been observed that combining CDK inhibitors with either conventional cytotoxic drugs or novel signal transduction modulators dramatically promotes neoplastic cell death in a variety of preclinical models. Efforts are underway to uncover inhibitors that selectively target specific CDKs and to develop these as a new generation of antitumour drugs. For all of these reasons, it is likely that interest in CDK inhibitors as antineoplastic agents will continue for the foreseeable future.

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Abbreviations

CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
IAP	inhibitor of apoptosis
P-TEF	positive transcription elongation factor
RNAPII	RNA polymerase II
VEGF	vascular endothelial growth factor

Introduction: rationale for the development of CDK inhibitors as antineoplastic agents

Cyclin-dependent kinases (CDKs) represent key molecules involved in regulation of the cell-cycle. CDKs are serine/threonine kinases that become active only when associated with a regulatory partner (e.g. cyclins or other proteins). CDK/cyclin holoenzymes are activated by phosphorylation, which is catalyzed by CDK-activator kinase (CAK). The activity of CDKs is negatively regulated by direct interactions with proteins referred to as CDK inhibitors (CDKIs). CDKIs are divided into two major families: the INK4 (inhibitor of CDK4) family, including $p16^{ink4a}$, $p15^{ink4b}$, $p18^{ink4c}$ and $p19^{ink4d}$, which specifically inhibit cyclin D-associated kinases (CDKs 4 and 6); and the Cip/Kip (kinase inhibitor protein) family, consisting of $p21^{cip1/waf1}$, $p27^{Kip1}$ and $p57^{kip2}$, which inhibit most CDKs. Dysregulation of molecules controlling the cell-cycle plays an important role in tumour pathogenesis. For example, alterations in the cascade involving CDK4, CDK6, cyclin D, INK4, pRb and E2F have been observed in more than 80% of human cancers. Genetic abnormalities of other CDKs (e.g. 1, 2 and 7), cyclins (e.g. A and E) and CDKIs (e.g. $p27^{Kip1}$ and $p21^{waf1}$) have also been reported in many human cancers. Moreover, enforced perturbations in CDKs and/or CDKIs can promote neoplastic transformation and/or enhance the carcinogenic properties of oncoproteins. Thus, CDKs represent very attractive targets for cancer therapy. More than 50 pharmacological CDK inhibitors have been described, some of which have potent antitumour activity. Furthermore, multiple CDK inhibitors are currently undergoing preclinical and clinical evaluation, and the search for new CDK inhibitors continues. Recent advances in the development of CDK inhibitors as antitumour drugs are reviewed below, with an emphasis on flavopiridol, the first CDK inhibitor to undergo clinical evaluation in humans.

Flavopiridol

Flavopiridol (NSC 649890, L86-8275 or HMR 1275) is a semisynthetic small molecular derivative of rohitukine, an alkaloid isolated from *Dysoxylum binectariferum*. Initially, flavopiridol demonstrated potent effects on cell proliferation ($IC_{50} = 66$ nM) in 60 National Cancer Institute human tumour cell lines, with no obvious tumour-type selectivity [1]. This effect has also been observed in xenograft models involving various tumour-types [2]. Mechanistically, the antitumour activity of flavopiridol has been related to CDK inhibition, induction of apoptosis, inhibition of transcription and anti-angiogenic activity.

Inhibition of CDKs by flavopiridol

Cell-cycle progression (i.e. the traverse through G1, S, G2 and M phases) is tightly controlled by CDK holoenzyme complexes consisting of catalytic subunits (CDKs) and regulatory subunits (cyclins) [3]. 13 CDKs and 25 cyclin-box-containing proteins have been identified through human genome sequencing. Among these, CDKs 1, 2, 4 and 6 and cyclins A, B, D and E are directly involved in regulating progression through the cell-cycle. The CDK7/cyclin H complex (i.e. CAK) is involved in cell-cycle

regulation through phosphorylation and activation of CDKs 1, 2, 4 and 6.

Flavopiridol inhibits several protein kinases, with the greatest activity directed against CDKs. In *in vitro* and *in vivo* studies, flavopiridol has been shown to inhibit CDK1/cyclin B (IC_{50} = 30–40 nM), CDK2/cyclin A and CDK2/cyclin E (IC_{50} = 100 nM), CDK4/cyclin D (IC_{50} = 20–40 nM), CDK6/cyclin D (IC_{50} = 60 nM) and CDK7/cyclin H (IC_{50} = 110–300 nM) [4]. Analysis of X-ray crystal structures revealed that L868276 (a deschlorophenyl derivative of flavopiridol) binds to the ATP binding pocket of CDK2 [5]. As the molecular structures of CDKs are similar (with 40% shared homology, including the highly conserved catalytic core region of 300 residues), it is likely that flavopiridol inhibits the activity of most CDKs by directly occupying the ATP binding site, a function competitively blocked by excess ATP. Indeed, in soluble cell extracts, CDKs 1, 2, 4 and 7 bind to immobilized flavopiridol only in the absence of ATP [4]. Furthermore, flavopiridol can inhibit CDK7/cyclin H, leading to interference with phosphorylation of specific threonine residues of most CDKs (e.g. Thr161 in cdc2/CDK1, Thr160 in CDK2, Thr172 in CDK4 and Thr177 in CDK6), an event that is required for their full activation. Inhibition of CDKs 1, 2 and 4 by flavopiridol leads directly to cell-cycle arrest at the G1/S and G2/M phase transitions, and a delay in S phase progression [1,2]. In tumour cells lacking CDK4, flavopiridol induces G1 arrest by inhibiting CDK6 [4]. Downregulation of cyclin D1 (see below) can also contribute to cell-cycle arrest following flavopiridol treatment. The patterns of flavopiridol-induced cell-cycle arrest (G1/S and/or G2/M arrest) appear to be cell type-specific.

Inhibition of transcription

The elongation phase in transcription of genes encoding eukaryotic proteins is controlled by RNA polymerase II (RNAPII), and is determined by the interplay between negative (N-TEF) and positive (P-TEF) transcription elongation factors. P-TEFb is the only known component of P-TEF. Human P-TEFb is composed of CDK9 and cyclin T (T1, T2a or T2b), forming a holoenzyme complex that phosphorylates (activates) the C-terminal domain of RNAPII [6]. Specific binding of the small nuclear RNA 7SK to P-TEFb blocks CDK9 activity [7]. In addition, other CDK complexes might also participate in transcriptional regulation by either phosphorylating the C-terminal domain of RNAPII (e.g. CDK9/cyclin K, CDK7/cyclin H/Mat1 and CDK8/cyclin C), regulating pre-mRNA splicing (CDK11/cyclin L), or modulating the transcripition factor Ets2 (CDK10) [8–10].

Flavopiridol potently blocks transcriptional activity of RNAPII *in vitro* by inhibiting P-TEFb (CDK9/cyclin T), a process initially related to inhibition of HIV replication (IC_{50} < 10 nM) [11]. Flavopiridol inhibits the C-terminal domain kinase activity of P-TEFb (K_i of 3 nM) more potently than other CDKs (e.g. CDKs 1, 2, and 4; K_i values of 40–70 nM). Furthermore, inhibition of CDK9 by flavopiridol is non-competitive with respect to ATP [12••]. Structural information indicates that flavopiridol binds to the ATP binding pocket of CDK9 with higher affinity than it does to CDK2 [13•]. *In vitro* and *in vivo*, flavopiridol inhibits transcription at concentrations much lower than those required for inhibition of other CDKs, even in the presence of ATP. Whether inhibition of CDK7 is involved in blocking transcription remains unknown, but it is clear that inhibition of CDK7 requires much higher concentrations of flavopiridol (IC_{50} > 100 nM) than those required for inhibition of transcription, at least in the case of HIV Tat (a potent transactivator of viral gene expression, which is essential for HIV-1 replication) transcription. DNA microarrays have demonstrated that flavopiridol inhibits gene expression broadly, closely mimicking the actions of two specific transcription inhibitors (actinomycin D and DRB [5,6-dichloro-1-beta-*D*-ribofuranosylbenzimidazole]), indicating that flavopiridol might act as a global transcription inhibitor [14]. However, it is unclear if the predominant effect of flavopiridol on gene expression results from its ability to inhibit P-TEFb, particularly in mammalian cell systems. The potent inhibitory effects on viral gene transcription raise the possibility that CDK inhibitors (e.g. flavopiridol and roscovitine) could be developed as clinical antiviral drugs [15].

One of the most interesting molecules to be transcriptionally downregulated by flavopiridol is cylcin D1. D-type cyclins (particularly D1) are multifunctional proteins that participate in cell-cycle regulation, cell growth and carcinogenesis [16]. Exposure of breast cancer cells to flavopiridol (100–300 nM) results in a decline in cyclin D1 promoter activity, leading to a decrease in cyclin D1 mRNA and protein levels [1]. *In vivo*, flavopiridol depletes cyclin D1 in tumour xenografts [2]. Transcriptional inhibition of cyclin D1 has been associated with cell-cycle arrest and possibly cell death following flavopiridol treatment. Such observations are consistent with the finding that flavopiridol produces a significant delay in disease progression in 84% of patients with mantle cell lymphoma — a tumour with overexpression of cyclin D1 in 95% of cases [17].

Induction of apoptosis

Apoptosis plays a pivotal role in drug- and radiation-induced cytotoxicity. Mechanistically, initiation of apoptosis involves at least two distinct pathways: extrinsic (death receptor-related) and instrinsic (mitochondria-dependent) pathways. Members of the Bcl-2 family, which includes both anti-apoptotic (e.g. Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl-1) and pro-apoptotic proteins (Bax family: Bax, Bak and Bok; BH3-only family: Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa and Puma), are important apoptotic regulators [18]. Inhibitors of apoptosis (IAPs;

e.g. XIAP, cIAP1, cIAP2, NAIP, MLAIP, ILP2, livin [KIAP], apollon and survivin) represent another regulatory protein family involved in apoptotic signaling, the members of which directly bind to and inhibit caspases 3, 6, 7 and 9 [19]. In turn, IAPs are inhibited by mitochondrial release of Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP binding protein with low pI released from mitochondria) [19].

In vitro, exposure to 100–400 nM flavopiridol for 6–48 hours induces apoptosis in a variety of tumour cells [4]. Human leukemia cells, independently of their origin and type, appear to be particularly sensitive to flavopiridol, as are multiple myeloma cells. Moreover, flavopiridol exerts lethal effects in tumour cells that are resistant to DNA-damaging agents and radiation [1]. *In vivo*, flavopiridol induces apoptosis in tumour xenografts, as detected by TUNEL assay, with significant reductions of up to 60–70% in tumour size [1]. The mechanisms responsible for flavopiridol-induced apoptosis are complex and probably multifactorial. Although cleavage of caspase-8 and Bid has been observed in tumour cells after flavopiridol treatment, it is unlikely that the extrinsic pathway represents the primary cell death trigger because flavopiridol is able to induce apoptosis in cells lacking caspase-8 [20]. Moreover, caspase-8 inhibitors (either pharmacological or viral) fail to block mitochondrial damage and apoptosis induced by flavopiridol [21].

Transcriptional inhibition of genes that encode anti-apoptotic regulators has been postulated to play a central role in the cytotoxicity of flavopiridol. For example, downregulation of Bcl-2 protein has been observed in several cell lines following flavopiridol treatment [22,23]. However, neither ectopic expression nor antisense-oligonucleotide-mediated downregulation of Bcl-2 exerts an effect on flavopiridol-induced cell death, indicating this cell death is largely independent of Bcl-2. Such findings may potentially explain why flavopiridol can overcome chemotherapeutic drug resistance secondary to Bcl-2 overexpression [20]. Interestingly, overexpression of an N-terminal phosphorylation-loop-deleted Bcl-2 protein provides a high degree of resistance to human leukemia cells treated with flavopiridol [24].

Recently, evidence has appeared suggesting that the anti-apoptotic protein Mcl-1 represents an important flavopiridol target. Firstly, decreases in Mcl-1 protein and/or mRNA levels have been observed in flavopiridol-treated tumour cell lines (e.g. lung cancer and multiple myeloma), freshly isolated tumour cells (i.e. from patients with B cell chronic lymphocytic leukemia and multiple myeloma) and in leukemic cells obtained from flavopiridol-treated patients [25,26••,27–29]. Secondly, downregulation of Mcl-1 has been related to accumulation of E2F-1 [28], disruption of signal transducer and activator of transcription-3 (STAT3)/DNA binding [29] and direct inhibition of C-terminal domain phosphorylation of RNAPII associated with flavopiridol treatment [26••]. Moreover, enforced expression of Mcl-1 provides a high degree of resistance to tumour cells against flavopiridol toxicity [26••,28]. In addition, downregulation of other anti-apoptotic proteins, including XIAP, BAG-1 and Bcl-xL, has been reported in certain tumour cells treated *in vitro* with flavopiridol [25,30].

Flavopiridol can also directly bind to duplex DNA [31], a finding that provides a possible explanation for the capacity of flavopiridol to kill noncycling (resting) cancer cells, as well as normal cells (e.g. peripheral lymphocytes, endothelial cells and embryonic neurons). However, normal cells appear to be less sensitive than tumour cells to flavopiridol-mediated lethality. It is unclear whether inhibition of CDK-mediated pathways is required for induction of apoptosis. In addition, there is a lack of evidence demonstrating that transcriptional repression of cyclin D1 by flavopiridol contributes functionally to its cytotoxicity, although antisense-mediated reductions in cyclin D1 expression can induce apoptosis in carcinoma cells [4].

Interestingly, administration of flavopiridol dramatically protects neurons form cell death induced by a variety of pathological stimuli. This phenomenon appears to be related to inhibition of CDK activity (CDK4/cyclin D1 and probably CDKs 1, 2 and 6) [32–34].

Synergism between flavopiridol and conventional cytotoxic agents

Cotreatment with flavopiridol and cytostatic drugs significantly increases cytotoxicity in a variety of tumour cells. In most cases, synergistic interactions are sequence-dependent, with administration of cytostatic drugs before flavopiridol yielding optimal lethality. Following the success of *in vitro* preclinical models, several clinical trials combining flavopiridol and various standard chemotherapeutic agents have been initiated (see below) [35,36]. The mechanisms underlying synergism appear to differ depending upon the cytostatic drugs used. Exposure of tumour cells to adriamycin and taxol results in a four- to five-fold increase in expression of survivin, whereas inhibition of p34cdc2/cyclinB-mediated survivin phosphorylation on Thr34 by flavopiridol results in diminished expression of survivin [37••]. These findings might explain why sequential administration of flavopiridol after such agents enhances apoptosis. In lung cancer cells, pretreatment with non-toxic concentrations of gemcitabine or cisplatin imposes an S-phase delay, which renders transformed cells selectively sensitive to the cytotoxicity of flavopiridol [38••]. Conversely, pre-treatment with flavopiridol can recruit leukemia cells into a proliferative state and thus 'prime' them for the S-phase-related cytotoxicity of nucleoside analogues such as ara-C [39]. Synergism following sequential treatment of tumour cells with gemcitabine and then flavopiridol might also be related to

ubiquitin/proteasome-dependent degradation of E2F-1, resulting in downregulation of the ribonucleotide reductase M2 subunit, which has been shown to contribute to gemcitabine resistance [40]. Flavopiridol selectively enhances doxorubincin-mediated cell death in cells exhibiting a functional defect in pRb, which is a common abnormality in human cancers [41]. Apoptosis induced by treatment with SN-38 (the active metabolite of camptothecin CPT-11) followed by flavopiridol is associated with cleavage of $p21^{cip1}$ and XIAP, and suppression of Drg1 expression, a novel gene responsible for resistance to CPT-11 [42,43]. Finally, recent studies suggest that sequence-dependent cytotoxicity of Epo-B (a non-taxene tubulin-polymerizing agent) followed by flavopiridol may be related to flavopiridol-induced Bax conformational change and downregulation of IAPs (XIAP, cIAP2 and survivin), Bcl-xL and Mcl-1 [44].

Synergism between flavopiridol and novel agents

There is increasing evidence that synergistic interactions also exist between flavopiridol and a variety of other novel signaling modulators. One interesting finding is that flavopiridol appears to convert drug-induced cell differentiation into apoptosis [45]. For example, simultaneous, but not sequential, exposure of leukemia cells to subtoxic concentrations of flavopiridol and the phorbol ester PMA (a potent inducer of differentiation) synergistically induces apoptosis and overcomes resistance mediated by overexpression of Bcl-2 [46•]. Such synergism is associated with abrogation of PMA-induced $p21^{cip1}$ expression by flavopiridol. These findings have now been extended to include other classes of differentiation-inducing agents, including inhibitors of histone deacetylase, such as suberoylanilide hydroxamic acid and sodium butyrate. Thus, cotreatment with flavopiridol blocks $p21^{cip1}$ induction by butyrate and suberoylanilide hydroxamic acid, leading to synergistic induction of apoptosis in multiple leukemia cell-types [47]. Such findings are consistent with earlier reports indicating that antisense-mediated inhibition of $p21^{cip1}$ expression blocks differentiation and increases leukaemic cell sensitivity to butyrate-mediated apoptosis [48]. In addition, synergistic interactions between flavopiridol and PMA or histone deacetylase inhibitors have been associated with downregulation of cyclin D1 and Mcl-1, cleavage of pRb and Bcl-2, and activation of E2F-1. Subsequent studies demonstrated that synergism between flavopiridol and PMA stemmed from PKC-dependent induction of tumour necrosis factor-α and activation of the extrinsic apoptotic pathway [49]. Analogously, the protein kinase C activator bryostatin 1 has also been shown to interact synergistically with flavopiridol through a similar tumour necrosis factor-α-related mechanism [50]. Consistent with this model, recent evidence has appeared indicating that flavopiridol interacts synergistically with TRAIL (TNF-related apoptosis-inducing ligand) [51]. Flavopiridol has also been shown to potentiate STI571-induced apoptosis in Bcr-Abl-positive leukemia cells, including those resistant to the Bcr/Abl kinase inhibitor STI571 (Gleevec, imatinib mesylate) [52]. Interestingly, synergism between flavopiridol and STI571 was associated with downregulation of Mcl-1, Bcl-xL and cyclin D1, and activation of Jun N-terminal kinase. Flavopiridol and herceptin (trastuzumab, anti-erbB2 antibody) interact synergistically in breast cancer cells in association with both inhibition of the Ras/MAPK/Akt pathway and diminished cyclin D1 expression [53,54]. Finally, cytotoxic synergy has been observed between flavopiridol and LY294002, a selective phosphatidylinositol 3-kinase inhibitor [54], suggesting an important role for the phosphatidylinositol 3-kinase/Akt pathway in protecting leukaemic cells from flavopiridol-mediated lethality. Collectively, these findings suggest that the concept of combining flavopiridol with other novel signaling modulators warrants further exploration as an anticancer strategy.

Other potential mechanisms of flavopiridol lethality

In several systems, flavopiridol has shown significant antiangiogenic activity. Such activity has been related to the ability of flavopiridol to induce apoptosis in endothelial cells through an unknown mechanism, which appears to be independent of CDK inhibition [4]. Another possibility is that flavopiridol acts through inhibition of vascular endothelial growth factor (VEGF) expression. For example, it has been shown that flavopiridol blocks hypoxia-induced VEGF mRNA transcription and decreases VEGF mRNA stability, leading to downregulation of VEGF protein [55].

Glycogen phosphorylase has been identified as a flavopiridol-binding protein [56]. Such direct binding could lead to the inhibition of glycogen phosphorylase. Indeed, flavopiridol treatment causes an increase in glycogen accumulation in lung cancer cells [57]. These findings raise the possibility that interference with glucose homeostasis may also contribute to the antineoplastic effects of flavopiridol.

Flavopiridol resistance

Resistance of tumour cells to chemotherapeutic drugs represents a major obstacle in anti-cancer therapy. It has been reported that flavopiridol fails to exhibit cross-resistance to most conventional cytotoxic agents, including etoposide, doxorubicin, vinblastine, oxaliplatin, paclitaxel, cisplatin, topotecan and 5FU [4]. More specifically, overexpression of mdr-1 (encoding PgP-170) or mrp-1 (encoding MRP-190), the major multidrug resistance proteins implicated in conferring resistance to natural product cytotoxic agents, does not cause appreciable resistance to flavopiridol [58–60]. Thus, the lack of cross-resistance between conventional cytotoxic agents and flavopiridol provides a basis for developing combination regimens incorporating flavopiridol and established cytotoxic agents.

Clinical trials

Phase I and II trials of flavopiridol have been completed involving two major schedules (i.e. a 72-hour infusion every two weeks, or a one hour infusion for five, three or one day every three weeks). In the 72-hour infusion regimen, steady-state plasma flavopiridol concentrations were 271–415 nM [1,2,35] whereas, in the one hour infusion regimen, the median post-infusion peak concentration was 1.7–3.8 μM [1,2,35]. These clinically achievable concentrations were significantly above those required to inhibit CDKs and cell growth, and induce apoptosis *in vitro*. In addition, there was evidence of antitumour activity against renal cell carcinoma, colon carcinoma, non-Hodgkin lymphoma, gastric carcinoma and especially mantel cell lymphoma [1,17]. However, the activity of flavopiridol administered alone has been less than that encountered in *in vitro* and xenograft model systems. Therefore, in addition to further phase I and II single-drug trials, efforts are underway to combine flavopiridol with other FDA-approved anti-cancer agents, including paclitaxel, fludarabine, cytosine arabinoside and irinotecan [35,36]. Attempts to combine flavopiridol with other signal transduction modulators are also under development.

Other CDK inhibitors

As a member of the first generation of CDK inhibitors, flavopiridol acts as a 'pan' CDK antagonist and, as such, is not particularly selective for a specific CDK. However, more selective CDK inhibitors have since been developed. Several of these compounds are being evaluated for antitumour activity in preclinical model systems.

Purine-based CDK inhibitors display greater selectivity for CDKs 1, 2 and probably 5, but exhibit no inhibitory activity toward CDKs 4 and 6. Some of these inhibitors have been reported to exert potent antitumour activity, including olomoucine, roscovitine, purvalanols and CGP74514A (a 2,6,9-trisubstituted purine).

Olomoucine and roscovitine

Olomoucine was one of the first CDK inhibitors to be developed, and selectively inhibits CDKs 1, 2, 5 and probably 7 (but not CDK4) at micromolar concentrations. It has been shown to exert 50% growth inhibition against a National Cancer Institute panel of 60 tumour cell lines (IC_{50} = 60.3 μM), typically arresting cells at the G1/S and G2/M transitions [5]. Roscovitine is an olomoucine derivative and shares similar selectivity for CDKs (i.e. CDKs 1, 2 and 5, but not CDKs 4 and 6). However, it displays more potent (5- to 10-fold greater) inhibitory activity towards CDKs (particularly CDK1), and increased antitumour activity (average IC_{50} = 16 μM for all 60 tumour cell lines) [5]. *In vitro*, roscovitine also inhibits CDK3, which is involved in cell-cycle regulation [61]. Roscovitine and olomoucine can also efficiently stabilize and activate nuclear p53 by suppressing MDM2 (murine double minute 2) expression, which might permit them to function as sensitizing agents for DNA-damaging drugs [62,63]. Higher concentrations of roscovitine can block synthesis of RNA (probably by inhibiting phosphorylation of the C-terminal domain of RNAPII in a similar manner to flavopiridol) and DNA (by direct effects on DNA synthesis machinery) [63,64].

Interestingly, purine-based CDK inhibitors, such as roscovitine, can also exert anti-apoptotic effects in the nervous system [33,65], where activation and/or subcellular translocation (from nuclei to cytoplasm) of CDKs (e.g. CDKs 2, 4 and 5) appears to be essential for induction of cell death. Moreover, roscovitine can prevent neuronal death in neurodegenerative diseases (e.g. Alzeimer's disease) [66,67], possibly by inhibiting CDK5/p25-catalysed phosphorylation of the tau protein and/or CDK1-mediated phosphorylation and extracellular accumulation of β-amyloid, which are both hallmarks of these diseases.

CYC202

CYC202 (*R*-roscovitine) has been reported to display increased potency compared with roscovitine, with CYC202 showing more selective inhibition of CDK2/cyclin E (IC_{50} = 100 nM) and enhanced antitumour activity (IC_{50} = 15.2 μM in a panel of 19 human tumour cell lines) [68]. Indeed, in xenograft animal tumour models, CYC202 has been shown to exert significant antitumour effects. Specifically, it has been shown to induce cell death rather than cell-cycle arrest, with a 45–62% reduction in tumour growth [68]. Phase I trials of CYC202 are currently underway, making this the second CDK inhibitor to enter the clinical arena.

Purvalanols

Purvalanol A and B, which are derivatives of olomoucine, exhibit potent and selective inhibition of CDKs 1 and 2 with 1000-fold greater inhibitory activity toward CDK2/cyclin A compared with olomoucine [5]. *In vitro* studies demonstrated that purvalanol inhibits cell growth (IC_{50} = 2.5 μM) and induces G2/M arrest. These effects are linked to inhibition of CDK1 and p42/p44 mitogen-activated protein kinase, both of which have been identified as intracellular targets of purvalanol [69]. Administration of purvalanol did not appear to trigger extensive activation of caspases; however, induction of apoptosis might be highly dependent upon the concentration of CDK inhibitors. For example, it has been shown that treatment of leukemia cells with low concentrations (1 μM) of the tri-substituted purine CGP74514A induces cell-cycle arrest, whereas higher concentrations (> 3 μM) trigger mitochondrial damage and cell death [70]. Similarly, analogues of olomoucine, including plant cytokinin analogues, exhibited either cell-cycle inhibitory effects or induction of apoptosis in a dose-dependent manner in leukemia cells [71].

Indolinones

Indolinone-based CDK inhibitors also selectively inhibit CDKs 1, 2 and 5. For example, SU9516, initially developed as a CDK2-selective inhibitor, displayed more potent inhibition of CDK2 (IC_{50} = 22 nM) and CDK1 (IC_{50} = 40 nM) than of CDK4 (IC_{50} = 200 nM). In colon carcinoma cells, administration of SU9516 resulted in inhibition of CDK2 and diminished pRb phosphorylation, accompanied by either G0/G1 or G2/M arrest and caspase-3-mediated apoptosis [72].

Paullones

The paullone class of CDK inhibitors displays a high degree of selectivity for CDKs 1, 2 and 5, but no activity towards CDK4/cyclin D1. Thus, the inhibitory spectrum of this class of CDK inhibitors is similar to that of purine-based compounds. Among members of this class, kenpaullone and its derivative alsterpaullone have been found to be the most active against CDK1/cyclin B and are competitive with ATP [73]. The antitumour potential of the paullone class CDK inhibitors remains to be evaluated.

UCN-01

UCN-01 (7-hydroxystaurosporine) was originally developed as a selective protein kinase C inhibitor. However, it was subsequently shown that at higher (IC_{50} = 300–600 nM) concentrations UCN-01 inhibits CDK1 (cdc2) and CDK2 *in vitro* [1], and therefore can be characterized as a CDK inhibitor. Nevertheless, other studies have shown that the antitumour activity of UCN-01 is primarily related to its ability to induce abrogation of the G2 or G1 checkpoints (IC_{50} ~50 nM), resulting in 'inappropriate' activation of CDKs (e.g. cdc2/CDK1) [1,74]. This appears to represent a consequence of inhibition of phosphorylation of Cdc25C by the kinase chk1 [75]. Interference with the G1 and G2 checkpoints appears to be responsible for synergistic interactions between UCN-01 and conventional cytotoxic agents (e.g. cisplatin, ara-C and gemcitabine) [76–78]. More recently, UCN-01 has been shown to inhibit Akt [79], although the contribution of this action to the antitumour activity of UCN-01 is unclear. Clinical trials of UCN-01, either alone or in combination with established cytotoxic drugs, have begun in humans, and have revealed an exceptionally long plasma half-life for the compound as a result of extensive binding to plasma α1-acidic glycoprotein [80].

The search for selective CDK inhibitors

CDK4/cyclin D represents a desirable target for drug discovery for a variety of reasons, including its frequent dysregulation in human neoplasia. However, the development of selective CDK4 inhibitors is more difficult than that of CDK2 inhibitors, primarily because the crystal structure of CDK4 has not been acquired. Nevertheless, CDK inhibitors derived from pyrimidines have been found to selectively inhibit CDKs 4 and 6. For example, PD0183812 has been identified as a highly selective CDK 4 and 6 inhibitor (IC_{50} = 8 nM for CDK4/cyclin D1; 7.1 nM for CDK6/cyclin D2; and 13 nM for CDK6/cyclin D3), which induces G1 arrest and blocks proliferation only in tumour cells expressing pRb, resulting in a loss of pRb phosphorylation [81]. CINK4, a triaminopyrimidine derivative, is another pyrimidine-based compound that specifically inhibits CDK4/cyclin D1 (IC_{50} = 1.5 μM) *in vitro*. It has been shown that CINK4 causes growth arrest at the G1 phase in tumour cells and normal cells, and slows tumour growth in xenografts of colon carcinoma cells [82].

Although no inhibitor has been identified that is absolutely selective for a single CDK, the search continues for natural products and structure-based synthetic or semi-synthetic compounds that will selectively target particular CDKs (e.g. CDKs 1, 2 and 4) [83]. However, because of the conservation of amino acids framing the CDK ATP-binding pocket and the high structural homology in the CDK family, it might be difficult to design single CDK-specific inhibitors. In this regard, three-dimensional structural models of CDKs should provide useful information for the design of novel CDK-selective inhibitors. For example, the crystal structure of CDK2 is well characterized and has been widely used for the development of CDK2-specific inhibitors. As a result, a new purine-based CDK inhibitor has been described that is 1000-fold more potent than the parent coumpound (K_i = 9 nM for CDK1, and 6 nM for CDK2) [84•]. Similar efforts are underway to develop CDK4-specific inhibitors by using a CDK4 mimic CDK2 protein [85]. Alternatively, new approaches, including affinity chromatography utilizing immobilized inhibitors, have been employed to characterize the intracellular targets and selectivity of a particular CDK inhibitor [86•]. One question that remains to be resolved is whether a high degree of selectivity for a particular CDK represents a desirable characteristic for CDK inhibitors designed to function as antineoplastic agents.

Conclusions

Recently, we have witnessed a tremendous increase in our understanding of the factors regulating the cell-cycle, the role of cell-cycle dysregulation in carcinogenesis and the relationship between cell-cycle events and apoptosis. These have, in turn, served as a powerful impetus for the development of CDK inhibitors as antineoplastic agents. To date, the CDK inhibitors flavopiridol and CYC202 have entered the clinical arena, and others are certain to follow. However, several issues remain to be resolved before such agents can fulfill their potential. These include defining the relationship between CDK inhibition and induction of apoptosis, determining whether selective inhibition of a particular CDK is a desirable goal, and optimizing the use of CDK inhibitors in combination with other agents, including both conventional and novel antineoplatic drugs. Given the accelerated pace

of discovery in the area of cell-cycle regulation, it is apparent that the development of CDK inhibitors will be the subject of intense interest for many years to come.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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